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Translation and assembly of CABYR coding region B in fibrous sheath and restriction of calcium binding to coding region A ☆

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Abstract

CABYR is a highly polymorphic, sperm flagellar calcium-binding protein that is tyrosine as well as serine/threonine phosphorylated during capacitation. Six alternative splice variants of human CABYR (I–VI) have previously been identified, involving two coding regions, CR-A and CR-B, separated by an intervening stop codon. It is presently unknown if proteins encoded by the predicted coding region B of CABYR are translated during spermiogenesis, where they localize, or which CABYR isoforms bind calcium. Immunofluorescent and electron microscopic studies using polyclonal antibodies generated to the recombinant c-terminal 198 aa CABYR-B localized the isoforms containing CABYR-B to the ribs and longitudinal columns of the fibrous sheath in the principal piece of the flagellum. Antisera to recombinant CABYR-A and CABYR-B proteins recognized distinct populations of CABYR isoforms encoded by either CR-A alone and/or CR-B as well as a common population of CABYR isoforms. Only the recombinant CABYR-A and not the CABYR-B bound calcium in vitro, which is consistent with the hypothesis that CABYR-A is the only form that binds calcium in sperm. These observations confirmed that, despite the presence of the stop codon in CR-A, splice variants containing CR-B are expressed during spermiogenesis and assemble into the fibrous sheath of the principal piece; however, calcium binding occurs only to those CABYR isoforms containing CABYR-A.

Keywords: CABYR; Calcium binding; Capacitation; Sperm flagella; Protein phosphorylation

Introduction

Mammalian sperm acquire the capacity to fertilize an oocyte by undergoing a series of physiological and biochemical changes in the female reproductive tract termed capacitation. This process results in two well-characterized end points: hyperactivation of the flagellum and acrosomal exocytosis in the sperm head (Austin, 1952; Katz and Yanagimachi, 1980). Although capacitation was discovered more than 50 years ago, the molecular mechanisms that underlie these events are still being elucidated. Signaling cascades accompany capacitation and involve a sperm-specific adenylyl cyclase, protein kinase A, tyrosine kinase(s), and a number of downstream phosphorylated protein substrates. Defined media containing BSA, HCO_3^- and Ca^{2+} can induce changes that mimic capacitation, resulting in increased cAMP levels, PKA activity, and tyrosine phosphorylation (Leclerc et al., 1996; Osheroff et al., 1999). To date, only a few of the sperm proteins that undergo phosphorylation during capacitation have been

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defined including the sperm flagellar proteins AKAP 3 (Mandal et al., 1999; Vijayaraghavan et al., 1999) and AKAP 4 (Carrera et al., 1994; Fulcher et al., 1995; Turner et al., 1998).

CABYR, a cytoskeletal protein associated with the flagellar fibrous sheath scaffold of sperm, is tyrosine (Naaby-Hansen et al., 2002) as well as serine/threonine phosphorylated (Ficarro et al., 2003) during capacitation. Protein blot overlays with calcium-45 have revealed that the 86-kDa acidic isoforms of CABYR strongly bind calcium (Fig. 2C and Naaby-Hansen et al., 2002), and that among sperm proteins, the CABYR signal is second only to the known calcium-binding protein calmodulin (Fig. 2C). Most of the calmodulin is found in the sperm head, although significant levels are also found in the flagellum (Jones et al., 1978; Feinberg et al., 1981; Tash and Means, 1982). However, CABYR is the only calcium-binding protein localized exclusively to the flagellum and is proposed to function in the calcium waves that accompany flagellar beating (Suarez et al., 1993).

From amplification and sequencing of human testicular mRNAs, the single copy, six exon *CABYR* gene, located on chromosome 18 at q12.2, is predicted to encode six forms of CABYR (I–VI). These include 5 spliced variants that, interestingly, involve two distinct coding regions, CR-A and CR-B. A stop codon followed by fifteen in-frame nucleotides separates CR-A from CR-B resulting in 493 and 198 aa reading frames, respectively (Naaby-Hansen et al., 2002). The syntenic mouse CABYR gene on chromosome 18A1 is similarly organized into six exons involving two coding regions with four individual CABYR transcripts identified (Sen et al., 2003). CABYR has been studied to date only in human and mouse sperm, where extensive charge and mass polymorphism has been noted in CABYR proteins resolved on 1- and 2-D gels.

Due to this extensive alternative splicing and resultant protein polymorphism, key questions regarding the structure and cell biology of CABYR isoforms remain unanswered. For example, it is presently unknown if proteins containing CR-B sequences are translated during spermiogenesis. Additionally, calcium binding has been detected to a tightly restricted subset of CABYR isoforms, the major cluster migrating in 2-D gels at 86 kDa, p*I* 4.5 (Fig. 2C). However, the exact sequences of the calcium-binding isoforms of CABYR remain undefined.

At CABYR-A's N-terminus lie sequences homologous to the RII subunit of protein kinase A, including the A Kinase Anchoring Protein (AKAP) binding domain, RII dimerization domain, and a putative calcium-binding EF-hand-like motif (aa 197–209). Found in a large number of calcium-binding proteins from organisms of diverse phyla, each EF-hand motif consists of a 12-residue loop flanked on either side by a 12-residue alpha helix. To date, a minimum of two EF hand motifs (helix–loop–helix) are found in all known EF hand proteins (Kretsinger and Kockolds, 1973; Ikura, 1996). The EF-hand-like motif in human CABYR has the highest similarity to the EF-hand motif in FCaBP, a flagellar protein from the protozoan parasite *Trypanosoma cruzi* (our unpublished data). The flagellar localization of FCaBP has suggested that this calcium-binding protein may be involved in motility of the parasite (Engman et al., 1989; Godsel and Engman, 1999) and raised the possibility that CABYR may have a similar mechanism for binding calcium and may also be associated with flagellar motility. However, unlike trypanosomal FCaBP (and other calcium-binding proteins), CABYR is distinguished by a single putative EF-hand-like motif, raising the question whether this non-canonical EF-hand motif is indeed responsible for the observed calcium binding.

Indeed, many calcium-binding proteins do not contain EF-hand motifs and bind calcium through co-ordination of several negative charges. Calcium-binding proteins such as calsequestrin, for example, contain a preponderance of acidic residues and possess very acidic p*I*s (Scott et al., 1988). CABYR is also an acidic protein (p*I*: 4.5) and becomes more acidic during capacitation through phosphorylation (Naaby-Hansen et al., 2002). Thus, the calcium-binding properties of human CABYR may be due to its EF-hand-like motif, acidic nature, phosphorylation status, or a combination of these properties.

Because of the complexity of CABYR isoforms, the existence of multiple splice variants, the unusual intervention of a stop codon in the CABYR coding region, and the fact that calcium has been demonstrated to bind most intensely to a distinct highly acidic cluster of charge variants migrating in 2-D gels at 86 kDa, p*I* 4.5 (Fig. 2C and Naaby-Hansen et al., 2002), efforts to elucidate the molecular mechanisms mediating calcium binding have required first a dissection of the CABYR isoforms responsible for calcium binding. The present studies establish three important aspects of CABYR cell biology: (1) that proteins encoded by the predicted coding region B of CABYR are produced during spermiogenesis; (2) that isoforms containing the CABYR-B co-assemble with CABYR-A in the fibrous sheath; and (3) CABYR-A containing isoforms are responsible for calcium binding.

Materials and methods

Cloning and sequencing of human CR-B and truncated CR-A cDNA

Gene-specific primers were designed to create a *Nco*I site at the 5′ end and a *Not*I site at the 3′ end of a polymerase chain reaction (PCR) amplicon encompassing coding region B using the human CABYR cDNA sequences AF295037 and AF295038. Primers (forward primer: 5′-CAT GCC ATG GCA ACA AGT GAA CGA GGA CAA-3′; reverse primer: 5′-ATA GTT TAG CGG CCG CGT TTT CAG TTT CTG CTT TGC GAC G-3′) were obtained from GIBCO BRL (Life Technologies, CA). In order to test whether or

not the putative EF-hand-like motif mediates the binding to calcium, a truncated version of the CR-A cDNA was also cloned and expressed. To clone the truncated CABYR CR-A variant (aa 219–493) from CABYR cDNA (Accession # AF088868), two primers CABYR 219F-*Nco*I (forward primer: CAT GCC ATG GTG GCT GCT CCT CTT GTG T) and CABYR 493R-*Not*I (reverse primer: ATA GTT TAG GCG GCC GCT TCA GCT GTT GAT TCC CCT T) were used. PCR was performed with 10 ng of human CABYR cDNA as a template to obtain the human CR-B cDNA using a program of one 2-min cycle at 94°C followed by 35 cycles of denaturation, annealing, and elongation at 94°C for 30 s, 50°C for 1 min, and 68°C for 2 min. Amplimers of 594 bp of CR-B and 825 bp of truncated CR-A CABYR nucleotide sequences were separated on a 1% NuSieve (FMC Bio-Products, Rockland, ME) agarose gel, cloned into the pCR 2.1-TOPO vector (Invitrogen, San Diego, CA), and sequenced in both directions to confirm authenticity, using vector-derived and insert-specific primers.

Expression and purification of the CR-B and CR-A recombinant proteins

The *Escherichia coli* strain BL21-SI (Invitrogen, Carlsbad, CA) was transformed with pET 28b+ vector containing the CR-B or truncated CR-A construct with six histidine residues at the C-terminus of the protein. A single colony was picked from a transformation plate, inoculated into 7 l of LBON medium (LB without NaCl) containing 50 μg/ml of Kanamycin, and grown at 30°C until the A_{600} reached 0.5. Recombinant protein expression was induced at 30°C for 3 h with 0.3 M NaCl. The cells were centrifuged at 10,000 × *g* for 30 min and then transferred and suspended in 1× binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) containing 100 μg/ml lysozyme and 8 M urea. After brief sonication on ice, the cells were recentrifuged at 15,000 × *g* for 20 min. The urea soluble supernatant was loaded onto a Ni^{2+}-activated His-binding resin column (Novagen, Madison, WI) following the manufacturer's protocol. The recombinant protein was eluted with 400 mM imidazole in 1× binding buffer containing 8 M urea, and precipitated with ethanol. Expression of a 22-kDa recombinant CABYR-B was confirmed on a Western blot using anti-histidine antibody. The purity of the isolated recombinant protein was confirmed by obtaining a single band of CABYR-B at the expected molecular weight (22 kDa) as detected in Coomassie and silver staining of 1-D gels.

Generation of anti CABYR-B antibody

The purified CABYR-B recombinant protein was stained in gel with Coomassie blue. The 22-kDa protein band was excised, homogenized in PBS, and emulsified with an equal volume of complete Freund's adjuvant. Approximately 30 μg of the affinity-purified recombinant protein, quantitated using BSA standards on the stained gel, was injected subcutaneously and intramuscularly into two sites on each of two female Sprague–Dawley rats. Animals were boosted three times at intervals of 14 days with 30 μg of recombinant protein in incomplete Freund's adjuvant and serum was collected 7 days after each boost. Rats were sacrificed to collect the antisera after confirming the presence of antibody by Western blot analysis on both the recombinant CABYR-B and on human sperm proteins. Generation of antibody to full-length recombinant CABYR-A has been previously reported (Naaby-Hansen et al., 2002).

Preparation of human sperm proteins

Proteins from "swim up" human sperm were extracted as previously described (Shetty et al., 1999). Sperm were solubilized by constant shaking for 1 h at 4°C in a Celis lysis buffer containing 9.8 M urea, 2% NP-40, 100 mM DTT, and the protease inhibitors: 2 mM PMSF, 5 mM iodoacetamide, 5 mM EDTA, 3 mg/ml L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride, 1.46 mM pepstatin A, and 2.1 mM leupeptin. Insoluble material was removed by centrifugation at 10,000 × *g* for 5 min, and the supernatant containing solubilized human sperm protein was subjected to one- and two-dimensional electrophoresis.

Electrophoresis

For one-dimensional electrophoresis, approximately 30 μg of sperm or bacterial lysates and 200 ng of purified recombinant CR-B protein were separated by 15% or 12.5% (w/v) SDS-PAGE (Laemmli, 1970) under reducing conditions. For two-dimensional electrophoresis, IEF gels were performed using Celis extraction buffer following methods previously described (Naaby-Hansen et al., 1997). Human sperm proteins were extracted in the presence of protease inhibitors and separated by isoelectric focusing (IEF) in acrylamide tube gels containing ampholine compositions of pH 3.5–5 (30%), pH 5–7 (20%), pH 7–9 (10%), and pH 3.5–10 (40%). 65 μl (approximately 200 μg determined by protein assay) of the sperm protein was applied to each IEF gel. Each tube was filled gently with an overlay buffer containing 5% NP-40, 1% ampholines (pH 3.5–10), 8 M urea, and 100 mM DTT. The gels were focused for 17,700 total volt-hours using voltage steps as follows: 200 V for 2 h, 500 V for 5 h, 800 V for 11 h, and 2000 V for 3 h. The IEF gels were then loaded on two-dimensional SDS-PAGE gels of 12.5% acrylamide (1.5 mm thickness, 16 × 20 cm). Electrophoresis was conducted at 240 mA for 4–5 h for six gels using a Protean II xi Multi-cell apparatus (Bio-Rad, Richmond, CA). Proteins were transferred onto nitrocellulose membranes using a BioRad Trans-Blot Cell as previously described (Naaby-Hansen et al., 1997).

Immunoblotting

Excess protein-binding sites on the membrane were blocked with PBS containing 5% (w/v) non-fat milk and 0.05% (w/v) Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany) for 1 h. The membrane was incubated overnight at 4°C with anti CABYR-B polyclonal antibody raised in rat or anti CABYR-A polyclonal antibody previously raised in rat (Naaby-Hansen et al., 2002). Anti-CABYR-A antibody was diluted 1:2000 and anti CABYR-B antibody was diluted 1:1000 with the blocking solution. Control pre-immune sera from each rat were used at the same dilutions as the immune sera for all experiments. Membranes were then incubated for 45 min with an anti-rat IgG-secondary antibody linked to horseradish peroxidase (Jackson Immuno Research Lab., West Grove, PA, USA), diluted 1:5000 in the blocking solution. The blot was incubated in SuperSignal West Pico peroxide and luminal/enhancer solution (Pierce, Rockford, IL) and then exposed to X-ray film (Eastman Kodak) or the blot was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (Kirkegaard and Perry lab., Gaithersburg, MD, USA).

Calcium overlay assay

The calcium-binding property of recombinant CABYR was demonstrated by an overlay assay using the radio-isotope calcium-45. The protocol followed a modified version of the method originally described by Maruyama et al. (1984). Briefly, proteins were subjected to SDS-PAGE analysis in a 12.5% gel and electrotransferred to PVDF membrane. The membrane was stained with Ponceau-S to highlight the protein load, define specific protein locations and confirm successful protein transfer, then photographed and washed several times in water to remove the stain. The membrane was incubated in washing buffer (10 mM imidazole HCl, 60 mM KCl, 5 mM $MgCl_2$, pH 6.8) for 45 min with buffer changes every 15 min. The washed membrane was incubated in 2 μCi of $^{45}CaCl_2$ per ml of the washing buffer for 30 min at room temperature. Subsequently, the membrane was washed for 2 min with distilled water and then for 30 s with 50% ethanol. The washed membrane was air-dried and exposed to phosphorimaging screens. The screen was scanned in the phosphorimager (Molecular Dynamics, Sunnyvale, CA) to obtain an autoradiograph of the blot. The ^{45}Ca overlay experiment was repeated 5 times with consistent results.

Indirect immunofluorescent localization of CABYR isoforms containing CABYR-B sequence on human sperm

Swim-up human sperm were washed in PBS containing 0.2 mM PMSF, diluted to a concentration of 1×10^6 sperm/ml, and then spotted on glass slides. The sperm were air-dried, and a subset was fixed with 4% paraformaldehyde for 30 min at room temperature. After washing 3 times in PBS, the samples were blocked in 10% normal goat serum in PBS overnight at 4°C. The sperm were then incubated with a 1:50 dilution of the anti CABYR-B antibody or its pre-immune serum (1:50 dilution) in the blocking solution for 2 h at room temperature. The slides were then washed 3×5 min in PBS, and incubated with the secondary antibody, FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch), at 1:100 dilution in 10% normal goat serum in PBS for 1 h at room temperature. The slides were washed 3×5 min in PBS, and Slow Fade-Light Antifade Kit (Molecular Probes, Inc.) was used to slow fluorescein quenching.

Electron microscopic localization

To avoid non-specific backgrounds, affinity purification of anti CABYR-B was performed using CABYR-B recombinant protein. The recombinant CABYR-B protein was separated on SDS-PAGE gels (Laemmli, 1970) and transferred to nitrocellulose membrane (Towbin et al., 1979). After staining with Ponceau S, the band containing CR-B recombinant protein was excised and incubated with anti-CABYR-B antibody. Bound antibody was eluted using an elution buffer containing 5 mM glycine (pH 2.3), 0.02% BSA (w/v), 0.5% Triton X-100, and 150 mM NaCl as described (Weinberger et al., 1985). The activity of the affinity-purified antibody was confirmed by Western analysis and used for the primary antibody incubation on electron microscopic grids. Swim-up human sperm were washed in PBS and processed as previously described (Naaby-Hansen et al., 2002). The grids were washed three times with PBS, and blocked in 10% normal goat serum in PBS for 30 min. Grids were incubated overnight at 40°C in pre-immune serum or affinity-purified anti-CABYR-B antibody diluted 1:5 in 10% normal goat serum in PBS. Following three times washing with PBS, grids were incubated in the blocking solution containing 10 nm gold-conjugated secondary antibody, goat anti-rat IgG (Goldmark Biologicals, Phillipsburg, NJ), diluted 1:50 in PBS. The grids were washed with distilled water four times, stained with uranyl acetate, and observed with a JEOL 100CX electron microscope (JEOL, Peabody, MA).

Results

Generation of recombinant CABYR-B protein and rat anti-CABYR-B polyclonal antibody

CABYR is a highly polymorphic protein originating from six alternative splice variants in human (I–VI) involving two coding regions CR-A and CR-B separated by an intervening stop codon. In order to characterize and to study the functions of these isoforms, it was important that antibodies specific to different CABYR isoforms be raised. A specific anti-CABYR-A polyclonal antibody was raised in rat (Naaby-Hansen et al., 2002). In order to generate antibody specific to CABYR-B, cDNA corresponding to human CABYR-B was expressed in bacteria. Recombinant

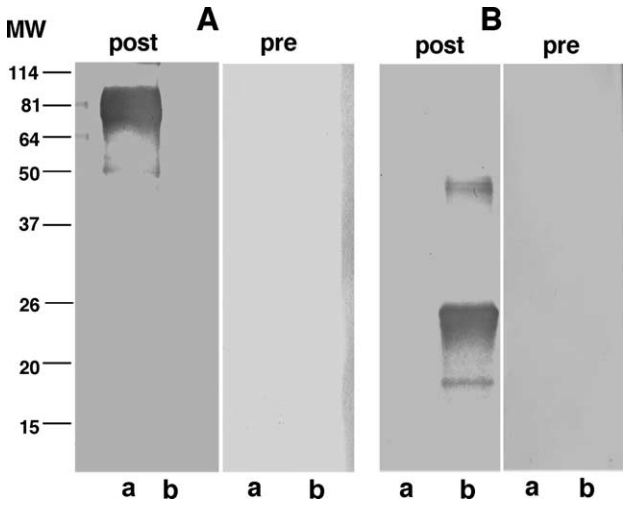

Fig. 1. Reactivity of anti CABYR-A antibody and anti CABYR-B antibody with recombinant CABYR-A and CABYR-B proteins. (A) Western blot reacted with rat anti-CABYR-A antibody shows immunoreactivity with CABYR-A recombinant protein (a), while CABYR-B recombinant protein (b) did not stain. Pre-immune serum from the identical rat did not react with either recombinant CABYR-A (a) or CABYR-B (b). (B) Western blot of rat anti-CABYR-B antibody reacted with only CABYR-B recombinant protein (b) and not with CABYR-A recombinant protein (a). Pre-immune serum did not react with either recombinant CABYR-A (a) or CABYR-B (b).

CABYR-B was subsequently purified and injected into rats to generate anti-CABYR-B antibody.

Antibody previously generated against CABYR-A (Naaby-Hansen et al., 2002) recognized the recombinant CABYR-A, but did not react with recombinant CABYR-B (Fig. 1A). Anti CABYR-B antibody detected the expected band at 22 kDa (Fig. 1B). However, it also detected two other bands in the affinity-purified recombinant CABYR-B preparation, albeit with less intensity (Fig. 1B). The lower band could be a degradation product of the CABYR-B. The higher molecular weight band appears to be a dimer, of CABYR-B. The detection of dimers was not expected because the disulfide links in the polypeptides are normally broken during preparation for SDS-PAGE analysis; however, the presence of some dimer might be due to incomplete reduction of the disulfide bridges in CABYR-B. Recently, CABYR has been shown to form dimers by virtue of the proline-rich extension-like domain (Hsu et al., 2005), which is situated in the CR-B region of CABYR (Naaby-Hansen et al., 2002). Pre-immune serum either for CABYR-A or CABYR-B did not detect any protein (Figs. 1A and B). Anti-CABYR-B antibody did not react with the recombinant CABYR-A (Fig. 1B). Thus, antibodies to CABYR-A and CABYR-B demonstrated specificity for the respective isoforms a finding essential for the ensuing studies.

Analyses of CABYR isoforms

Calcium overlay of 2-D blots of whole sperm extracts identified calcium binding to a tight cluster of CABYR isoforms at 86 kDa (Fig. 2C). On similar 2-D Western blots of whole sperm extracts, antiserum to the different coding regions recognized populations of CABYR isoforms that contained CABYR-A only, CABYR-B only, or epitopes from both coding regions (Figs. 2A and B). The isoforms containing CABYR-B sequences (Fig. 2B) were relatively less acidic (p*I*: 5–6.5) than those containing only CABYR-A (p*I*: ~4.5) (Fig. 2A). The 86-kDa calcium-binding forms of CABYR were detected only with anti CABYR-A antibody indicating that the CABYR isoforms with calcium-binding activity always contain sequence from CABYR-A. Furthermore, CABYR-B sequences did not contribute to the calcium-binding property of CABYR (Figs. 2B and C).

Calcium-binding isoform(s) of CABYR

Different amounts of recombinant CABYR-A and CABYR-B were used along with calmodulin (a known calcium-binding protein) and bovine serum albumin (a protein that does not bind to calcium) in calcium overlay experiments (Fig. 3). CABYR-A strongly bound calcium (Fig. 3A, lanes 1 and 2), whereas CABYR-B did not show

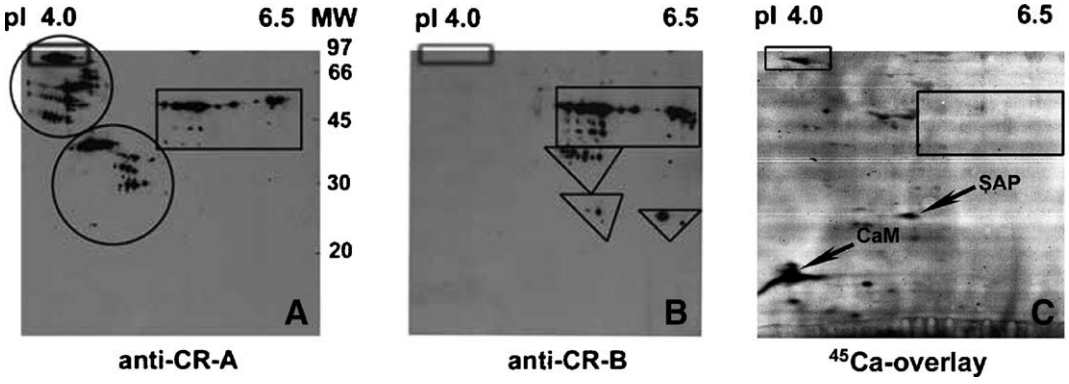

Fig. 2. Two-dimensional Western blots of anti CABYR-A antibody (A), anti CABYR-B antibody (B), and calcium-45 overlay (C) on the human sperm proteins. Circles indicate CABYR isoforms that reacted only with anti CABYR-A antibody. Triangles indicate forms that bound only to anti-CABYR-B antibody (likely peptide fragments). Large boxed areas indicate isoforms that contain both CABYR-A and CABYR-B sequences. In the small box, the 86-kDa isoforms of CABYR that bind calcium are seen to react only with anti CABYR-A. Calmodulin (CaM), serum amyloid P-component precursor (SAP), and 86 kDa CABYR-A were among the sperm proteins that bound calcium-45 in the calcium overlay assay (C). CABYR-B did not bind to calcium-45 (C).

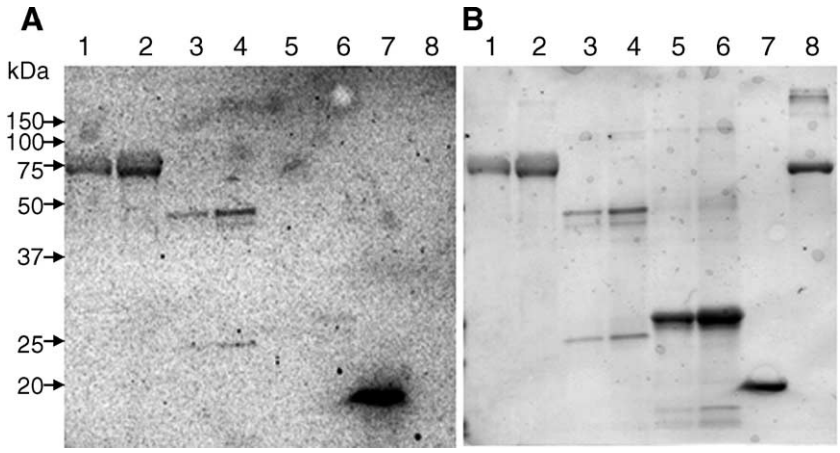

Fig. 3. Calcium-45 overlay assay of human recombinant CABYR-A and CABYR-B proteins. (A) Autoradiograph showing calcium binding [1.0 μg CABYR-A (lane 1), 2 μg CABYR-A (lane 2), 1.0 μg truncated (aa 219-493) CABYR-A (lane 3), 2.0 μg truncated CABYR-A (lane 4), 1.5 μg of CABYR-B (lane 5), 3.0 μg CABYR-B (lane 6), 1.5 μg calmodulin (lane 7, the positive control) and 5.0 μg BSA (lane 8, the negative control)]. (B) Ponceau-S stained blot before the overlay assay using calcium-45 radioisotope.

calcium binding even at higher protein concentrations than that of CABYR-A used in the overlay assay (Fig. 3, lanes 5 and 6). As expected, calmodulin showed very strong calcium binding (Fig. 3, lane 7) and BSA did not show any binding to calcium-45 (Fig. 3, lane 8), thus validating the assay conditions with positive and negative controls.

CABYR-A has a putative EF-hand-like domain at residues 197–209 which may mediate calcium binding. To further investigate the mechanism by which CABYR-A binds to calcium, a truncated version of recombinant CABYR-A (aa 219–493) without the putative EF-hand-like motif was expressed, purified, and compared to the full-length CABYR-A for its calcium-binding property. The truncated CABYR-A bound calcium-45 (Fig. 3, lanes 3 and 4) as did the full-length CABYR-A indicating that the putative EF-hand-like motif present in the CABYR-A is not essential for calcium binding.

Localization of isoforms containing CABYR-B sequence in human sperm

Indirect immunofluorescence analysis of human swim-up sperm using anti-CABYR-B antibody localized the isoforms containing CABYR-B sequences specifically to the entire principal piece of the flagellum with no staining in the midpiece, endpiece, or in the head (Fig. 4B). Pre-immune

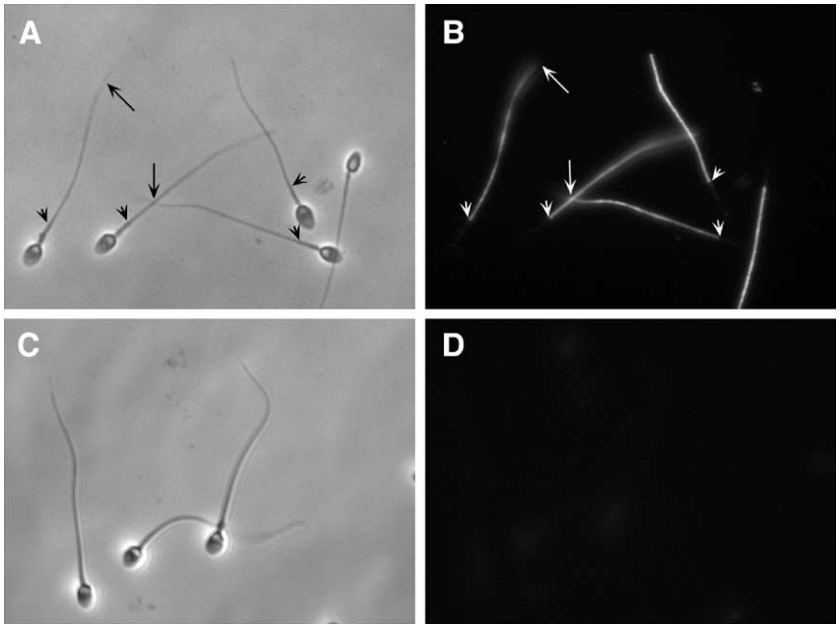

Fig. 4. Immunofluorescence localization of isoforms containing CABYR-B sequences to the principal piece of the human sperm flagellum. Phase-contrast (A, C) and fluorescence (B, D) micrographs of the same fields of human spermatozoa. When probed with anti-CABYR-B antibody, immunostaining is seen only in the principal piece of the flagellum (B). Pre-immune serum did not stain the human sperm (D). Long arrows indicate the posterior end of the principal piece whereas the short arrows indicate the posterior end of the midpiece. Original magnification is ×400.

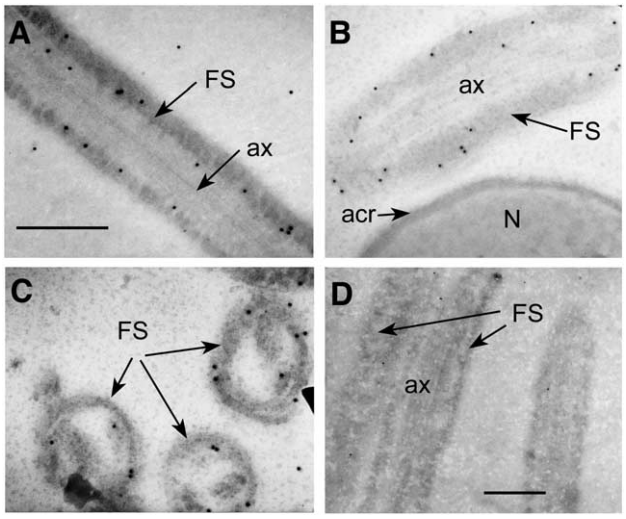

Fig. 5. Immunolocalization of isoforms containing CABYR-B sequences at the ultrastructural level showing that the anti-CABYR-B antibody was localized at the longitudinal columns and transverse ribs of the fibrous sheath of sperm tail (A, B, C). No significant staining was found in the fibrous sheath (FS) and axoneme (ax) by pre-immune serum (D). The scale bar is 0.5 μm.

serum showed no immunofluorescence in human sperm (Fig. 4D). Similar localization to the principal piece was reported with anti-CABYR-A antibody (Naaby-Hansen et al., 2002) indicating that the isoforms containing CABYR-B sequence co-localize in the principal piece with the isoforms containing CABYR-A. EM immunogold studies with anti-CABYR-B antibody localized isoforms containing CABYR-B sequence in the longitudinal columns and ribs of the fibrous sheath (Figs. 5A–C). There was no labeling in other components such as the mitochondrial sheath, axoneme, outer dense fibers, or nucleus (Figs. 5A–C). Sections exposed to pre-immune serum were unstained by immunogold (Fig. 5D).

Discussion

CABYR isoforms containing CABYR-B sequence are translated during spermiogenesis and incorporate into the flagellum

Naaby-Hansen et al. (2002) identified six human CABYR testicular mRNAs, CABYR I–VI, including 5 splice variants involving two coding regions, coding region A (CR-A), and coding region B (CR-B). A stop codon at the end of CR-A was followed by fifteen in-frame nucleotides that separated CR-A from the start codon of CR-B, resulting in 493 and 198 aa reading frames, respectively. Three CABYR splice variants were predicted to contain all 198 (III, V) or only 61 (VI) CR-B amino acids (Naaby-Hansen et al., 2002). At the time of submission of the present paper, GenBank contained 67 ESTs for human CABYR. Within this data set, no additional variant, beyond the previously reported CABYR I–VI, has been detected, although there are 104 human CABYR single nucleotide polymorphisms (SNPs) in the current GenBank (dbSNP build 124). Thus, the parts' list underlying CABYR protein polymorphism is now known. The present biochemical and morphological studies confirm that, despite the presence of the stop codon in CR-A, CR-B is expressed and translated during spermiogenesis, and that isoforms containing CABYR-B sequences incorporate into the sperm flagellum.

CABYR-B and CABYR polymorphism

The considerable polymorphism of immunoreactive CABYR isoforms on 2-D Western blots has been one of the most intriguing and perplexing aspects of CABYR molecular biology. Underlying the present experiments was the assumption that the polyclonal immunoreagents to full-length recombinant CABYR-A and/or CABYR-B would recognize epitopes located on all the six known human CABYR variants. These immunoreagents did indeed recognize distinct populations of CABYR on Western blots of 2-D gels, yielding findings that coincided in part with predictions based on CABYR sequence variants while leading to several new conclusions.

Compared to isoforms encoded by both CR-A and CR-B, the most acidic CABYR isoforms in sperm contain only CABYR-A. The splice variants I, II, and IV are predicted to encode CABYR-A proteins of 53, 51, and 31 kDa with p*I*s of 4.5, 4.5, and 4.2, respectively. The observation of an immunoreactive acidic family of CABYR-A isoforms is thus in agreement with the predicted charges of the isoforms encoded by the variants I, II, and IV. The slower migration of the highest CABYR-A isoforms (as high as 86 kDa) is likely due to their acidic nature since the recombinant form of full-length CABYR-A also migrated at approximately 86 kDa. The basis for the more acidic nature of CABYR-A isoforms than predicted is likely a combination of serine/threonine and tyrosine phosphorylation (Naaby-Hansen et al., 2002; Ficarro et al., 2003).

The family of isoforms that contain both CABYR-A and CABYR-B sequences migrates lower and is distinctly more basic than those containing only CABYR-A sequence. This is in agreement with the lower masses and more basic charges predicted for the isoforms encoded by the variants III, V, and VI.

The presence of relatively basic low molecular weight CABYR clusters that react only with anti CABYR-B antibody is an unexpected finding. No CABYR proteins are predicted to contain only CABYR-B sequences based on all available cDNAs. Further, overexposure of Northern blots of human testis RNAs did not detect any major CABYR transcript lower than 1.4 kb (Naaby-Hansen et al., 2002). Thus, in the absence of any evidence for truncated CABYR messages, the low molecular weight CABYR clusters containing only CABYR-B sequences likely result from proteolytic processing of isoforms encoded by the variants III, V, and VI or by translation of CR-B alone that

is mediated by a yet unidentified internal ribosomal entry site [IRES]. Since the stop codon present between the two coding regions is indeed a functional stop codon (our unpublished data), we hypothesize that translation of the CR-B (without a part of CR-A) occurs because of the presence of an IRES (Internal Ribosomal Entry Site) upstream to the start site of the CR-B and hence it is a 5′ cap-independent translation. Internal Ribosome Entry Sites are *cis*-acting RNA sequences able to mediate internal entry of the ribosomal subunit (40S) on some eukaryotic and viral mRNAs upstream of a translation initiation site. Recently, a number of novel IRES sequences have been discovered and examples continue to be added to the IRES database (Bonnal et al., 2003). IRES sequences are very diverse; for example, an IRES can be as few as 9 nucleotides or it can be very long (600 nucleotides). To constitute an IRES, it is believed that the primary RNA sequence should give rise to a certain RNA structure or it should contain binding sites for the *trans*-acting factors that will help recruit the ribosome. However, whether or not a particular mRNA has an IRES can be determined only on the basis of experimental evidence that it is able to translate a reporter placed in tandem to its 3′ end. It will be interesting to determine whether the 15 nucleotides that intervene between CR-A and CR-B of CABYR contain an IRES sequence which can mediate the translation of isoforms comprised of CR-B alone.

Isoforms containing only CABYR-A bind calcium

By radioactive calcium overlay, calcium binding was demonstrated to occur to the tight cluster of 86-kDa CABYR isoforms that underwent tyrosine phosphorylation during in vitro capacitation (Naaby-Hansen et al., 2002). It may now be concluded that CABYR-A alone is responsible for calcium binding to the 86-kDa forms of CABYR, and that CABYR-B sequences are not involved. Three lines of evidence support this conclusion. (1) The 86-kDa calcium-binding protein spot was cored from 2-D gels on three occasions, trypsinized, and microsequenced by mass spectrometry, and only CABYR-A microsequences were obtained. (2) No isoforms within protein clusters containing both CABYR-A and CABYR-B sequences have been shown to bind calcium on Western blots. (3) Examination of calcium interaction with both recombinant CABYR-A (encoded by the variant I) and CABYR-B revealed that only recombinant CABYR-A bound calcium.

Of the six CABYR variants, I, II, and IV do not contain any CR-B. The variants I and IV contain the sequence encoding a putative EF-hand-like motif; however, that sequence is spliced out in variant II. If calcium bound to these isoforms, it would be of considerable interest since the EF-hand-like domain is non-canonical and occurs singly. However, our data indicate that the non-canonical EF-hand-like domain is not involved in calcium binding to CABYR. First, no isoform within the clusters encoded by both CR-A and CR-B were shown to bind calcium and these clusters contain variable amounts of the first 180 amino acids of CABYR, so this domain can be tentatively excluded as the calcium-binding site. Second, after deleting the N-terminal 218 amino acids (that includes the EF-hand-like domain) from CR-A, calcium binding persisted in the truncated CABYR-A (aa 219–493). The predicted p*I* of the truncated protein is very acidic suggesting that calcium binds to isoforms containing CABYR-A sequences by virtue of their high negative charge. With CABYR-A established as the calcium-binding isoform, fine dissection of the calcium-binding motif is now possible.

Isoforms containing CABYR-A and CABYR-B sequences co-localize in the fibrous sheath

Immunofluorescence microscopy localized isoforms containing CABYR-B only in the principal piece of the human spermatozoon. This finding was consistent with fine structural immunolocalization of these isoforms to the ribs and columns of the fibrous sheath. Together with the previous work that localized isoforms containing CABYR-A sequences to the fibrous sheath (Naaby-Hansen et al., 2002), it may be concluded that multiple isoforms of CABYR assemble in this cytoskeletal element.

The polymorphism of CABYR and co-localization of CR-A and CR-B observed in the present study as well as motif analysis of CABYR splice variants lead to the postulate that CABYR isoforms oligomerize, possibly forming both homopolymers and heteropolymers. In fact, recently, Hsu et al. (2005) demonstrated that CABYR variants formed a dimer with a proline-rich extensin domain and that this domain overlapped with the GSK3β-binding site. Such a macromolecular assembly of CABYR isoforms with other signaling molecules may have evolved to tranduce signals in a specific and rapid manner in flagellum of sperm. Five PXXP consensus motifs, the cognate sites for SH3 interaction, are present in CABYR-A (aa 144–147, 153–156, 396–399, 471–474, and 473–476) and three are present in CABYR-B (aa 211–214, 214–217, 326–329). The crystal structures of several SH3 domains have been determined (Musacchio et al., 1992; Kohda et al., 1993), and the view has emerged that SH3 domains facilitate binding to partner proteins by interaction with cognate proline-rich regions containing the consensus sequence Pro-X-X-Pro (PXXP) (Musacchio et al., 1992; Kang et al., 2000). The consensus PXXP motifs present in CABYR may facilitate interactions of CABYR with the kinase that phosphorylates it or with other proteins. An Src homology domain 3 (SH3) is present in the non-catalytic part of several tyrosine kinases (e.g., Src, Abl, Lck) and it is believed that SH3 mediates the assembly of specific protein complexes via binding to proline-rich peptides. Thus, SH3 domains are thought to act as protein-binding structures and may be involved in

linking signals transmitted from the cell surface by protein tyrosine kinases (Olivier et al., 1993; Pawson, 1995; Pawson and Schlessinger, 1993). Considering other potential CABYR motifs for self-assembly, it should be noted that the sequence Val^{10}-Leu^{44} of CABYR-A bears 40% identity and 57% similarity to amino acids 7–41 of RIIα, the regulatory subunit of type II cAMP-dependent protein kinase (Newlon et al., 1999). This amino terminal region of RII contains both the RII dimerization domain and the AKAP binding domain (Newlon et al., 1999). Thus, CABYR isoforms contain structural modules that may participate in CABYR assembly in the fibrous sheath and in interactions with other proteins. The precise intermolecular details of CABYR self-assembly are among the most interesting questions for further exploration in the CABYR field.

Motility is a characteristic function of the sperm flagellum, permitting the male gamete to actively swim to and penetrate the egg in organisms with both internal and external fertilization. Mammalian sperm motility is acquired under the control of many extrinsic and intrinsic factors and is based on the specialized structure of components such as fibrous sheath, outer dense fibers, axonemal complex, and mitochondrial sheath. The sperm's fibrous sheath is among the most structurally and functionally interesting of complex cytoskeletal assemblies. This cytoskeletal element comprises two longitudinal columns connected by numerous transverse ribs and is composed of multiple protein SDS-PAGE bands in human (Jassim et al., 1992; Kim et al., 1997), rat (Oko and Clermont, 1989; Kim et al., 1995), and mouse (Eddy et al., 1991). The fibrous sheath proteins AKAP 3 (Mandal et al., 1999; Vijayaraghavan et al., 1999) and AKAP 4 (Carrera et al., 1994; Fulcher et al., 1995; Turner et al., 1998) are phosphorylated during sperm capacitation (Ficarro et al., 2003) and are thought to function as a scaffold for glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase [GAPDH in human (Westhoff and Kamp, 1997; Welch et al., 2000) and GAPDS in mouse (Bunch et al., 1998)] and hexokinase 1 (Mori et al., 1998; Travis et al., 1998). The fibrous sheath is also thought to form a scaffold that anchors constituents of signaling cascades including the Rho signaling pathway through a ropporin (Fujita et al., 2000) and rhophilin (Nakamura et al., 1999).

As a novel calcium-binding protein associated with the fibrous sheath, our model of CABYR function postulates a role in a calcium signaling pathway that regulates sperm motility during sperm capacitation. In support of this hypothesis is the observation that the acidic calcium-binding isoform of 86 kDa CABYR was increased during in vitro sperm capacitation and was abolished by dephosphorylation with alkaline phosphatase, suggesting that CABYR may be involved in the process of capacitation and motility of sperm (Naaby-Hansen et al., 2002). CABYR has an N-terminus motif similar to the regulatory subunit (RII) of cAMP-dependent protein kinase (PKA). Since the RII subunit of PKA is known to bind to A-Kinase Anchoring Proteins (AKAPs) which anchor PKA to specific sub-cellular locations within the cell (Colledge and Scott, 1999), AKAP binding might also be one of the functions of CABYR. Recognition that isoforms containing both CABYR-A and CABYR-B sequences localize in the fibrous sheath opens the way for use of both protein domains to identify binding partners that interact and assemble in this interesting cytoskeletal element.

It is noteworthy that recently two isoforms, CABYR III (379 aa) and CABYR V (281 aa), both of which include regions of CR-B, have been reported from tissues other than testis (fetal brain and adult brain, pancreas, and lungs) as well as brain tumors and cancer cell lines (Hsu et al., 2005). CABYR I (493 aa), the calcium-binding form, is suggested to be testis-specific (Hsu et al., 2005), a finding in accord with the post-meiotic localization of CABYR to spermatids using antisera to recombinant CR-A (Naaby-Hansen et al., 2002). In addition, ESTs of CABYR are also present in other tissues such as ovary, muscle, heart, lung, spleen, and retina. Therefore, CABYR may also be involved in other cellular functions in various tissues.

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